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Comparative Genomics and an Insect Model Rapidly Identify Novel Virulence Genes of *Burkholderia mallei*▽†

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***Burkholderia pseudomallei* and its host-adapted deletion clone *Burkholderia mallei* cause the potentially fatal human diseases melioidosis and glanders, respectively. The antibiotic resistance profile and ability to infect via aerosol of these organisms and the absence of protective vaccines have led to their classification as major biothreats and select agents. Although documented infections by these bacteria date back over 100 years, relatively little is known about their virulence and pathogenicity mechanisms. We used in silico genomic subtraction to generate their virulome, a set of 650 putative virulence-related genes shared by *B. pseudomallei* and *B. mallei* but not present in five closely related nonpathogenic *Burkholderia* species. Although most of these genes are clustered in putative operons, the number of targets for mutant construction and verification of reduced virulence in animal models is formidable. Therefore, *Galleria mellonella* (wax moth) larvae were evaluated as a surrogate host; we found that *B. pseudomallei* and *B. mallei*, but not other phylogenetically related bacteria, were highly pathogenic for this insect. More importantly, four previously characterized *B. mallei* mutants with reduced virulence in hamsters or mice had similarly reduced virulence in *G. mellonella* larvae. Site-specific inactivation of selected genes in the computationally derived virulome identified three new potential virulence genes, each of which was required for rapid and efficient killing of larvae. Thus, this approach may provide a means to quickly identify high-probability virulence genes in *B. pseudomallei*, *B. mallei*, and other pathogens.**

Burkholderiaceae is a family of the betaproteobacteria that colonize a variety of ecological niches. Some species are plant or animal pathogens, while others are important environmental bacteria, including nitrogen-fixing symbionts, plant growth-promoting rhizobacteria, chemolithoautotrophs, and bioremediation agents. Nearly all members of the *Burkholderiaceae* have large (5- to 9-Mb), multireplicon genomes (http://www.genomesonline.org/) comprised of a large core of orthologous genes and smaller subsets of species- or isolate-specific genes for ecological specialization. *Burkholderia pseudomallei* and *Burkholderia mallei* are aggressive human pathogens (7–9, 13) categorized as select agent biothreats with a high potential for misuse (3, 55, 67). The genome sequences of several strains of *B. mallei* and *B. pseudomallei* have revealed that *B. mallei* is a deletion clone of *B. pseudomallei* (27) which lost >1,000 genes. Many of the gene deletions appear to have been caused by insertion sequence-mediated events (49) and likely explain the many physiological differences that led to the classification of these two pathogens as separate species (54). Nearly all genes retained by *B. mallei* share ~99.5% DNA-DNA sequence identity with their *B. pseudomallei* homologs.

B. pseudomallei, which causes melioidosis, is an endemic and opportunistic pathogen that inhabits the tropical soils and waters of Southeast Asia and Northern Australia (2, 8, 58, 70), whereas *B. mallei* appears to be a zoonotic pathogen that infects a variety of animals, including equines and humans. The disease caused by *B. mallei*, glanders, is not well studied as there have been few well-documented cases since 1950. For both pathogens, infection usually occurs through wounds, aspiration, and possibly inhalation. Studies of intraperitoneally infected animals have shown that *B. pseudomallei* and *B. mallei* can rapidly migrate to the spleen and liver, where they multiply extensively within membrane-bound phagosomes and form abscesses (25, 37). Untreated infections are often fatal.

Small-animal models are available for studying the pathogenesis of *B. pseudomallei* and *B. mallei* (15, 34, 40, 42, 68). These models include a sensitive (50% lethal dose, <10 cells) Syrian hamster model utilizing intraperitoneal injection and two mouse models utilizing C57BL/6 or BALB/c mice and aerosol, intranasal, or intraperitoneal infection (40, 42). However, these models are expensive, cumbersome, and hazardous due to biosafety and biosecurity issues. A *Caenorhabditis elegans* nematode infection model for *B. pseudomallei* has been described (24, 50, 59), although it may have limited sensitivity due to the extremely high doses required for killing.

Compared to other bacterial pathogens, little is known about the virulence factors of *B. mallei* and *B. pseudomallei* or the molecular basis of the pathogenicity of these organisms. The only verified virulence factors for these pathogens are those that are well known and shared by most, if not all, animal and plant pathogens: exopolysaccharide capsule (CAP) (14, 53), type III secretion systems (T3SS) (19, 20, 23, 60, 64, 66), and lipopolysaccharide O antigen (LPS) (12). Type II secretion, type IV pili, and flagella (10, 11, 18) have been implicated in *B. pseudomallei* pathogenesis. Recently, a type VI secretion

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14. ABSTRACT

Burkholderia pseudomallei and its host-adapted deletion clone Burkholderia mallei, cause the potentially fatal human diseases melioidosis and glasnders, respectively. Their antibiotice resistance profile, ability to infect via aerosol, and the absence of protective vaccines has led to their classification as majjor biothreats and select agents. Although documented infections by these bacteria date back over 100 years, relatively little is known about their virulence and pathogenicity mechanisms. We used in silico genomic subtraction to generate their virulome, a set of 650 putative virulence-related genes shared by B. pseudomallei and B. mallei, but absent from five closely related nonpathogenicv Burkholderia species. Although most of these genes are clustered in putative operons, the number of targets for mutant construction and verification of reduced virulence in animal models is formidable. Therefore, Galleria mellnonella (wax moth) larvae were evaluated as a surrogate host; we found that B. pseudomallei and B. mallei,but not other related bacteria, were highly pathogenic in this insect. More importantly, four previously characterized B. mallei mutants with reduced virulence in hamsters or mice were similarly reduced in virulence on G. mellnella larvae. Site-specific inactivation of select genes from the computationally derived virulome identified three potentially new virulence genes, each of which were required for rapid and efficient killing of larvae. Thus, this approach may provide a means to quickly identify high-probability virulence genes in B. pseudomallei, B. mallei, and other pathogens.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Reference or source
Strains		
B. mallei ATCC 23344	Wild-type pathogen, Pm^r Km^s Gm^s Zeo^s	49
E. coli TOP10	General cloning strain	Invitrogen
E. coli S17-1	Tra^+ Sm^r Pm^s	57
B. mallei DD3008	Capsule-deficient mutant	14
B. mallei RD01	$T3SS_{AP}$-deficient mutant	64
B. mallei derivatives[b]		
GRS 23344	Δ*sacB* sucrose-resistant derivative	This study
Ilv	BMA1848 inactivated with pCRXL-*ilvI*; *ilv* auxotroph	This study
GspD	BMA2786 inactivated with pLCL9; type II secretion system mutant	This study
A0235	BMA0235 inactivated with pCRXL-A0235	This study
AA1900	BMAA1900 inactivated with pCRXL-AA1900	This study
AA1204	BMAA1204 inactivated with pCRXL-AA1204	This study
AA1013	BMAA1013 inactivated with pCRXL-AA1013	This study
A1123	BMA1123 inactivated with pCRXL-A1123	This study
AA1111	BMAA1111 inactivated with pCRXL-dicty	This study
AA1785	BMAA1785 inactivated with pCRXL-chitin	This study
A0847	BMA0847 inactivated with pCRXL-A0847	This study
Δ1517	BMAA1517 deletion in GRS 23344	This study
Δ1621	BMAA1621 deletion in GRS 23344	This study
Plasmids		
pCR-XL-TOPO	Cloning vector, Km^r Zeo^r	Invitrogen
pCR2.1-TOPO	Cloning vector, Km^r Amp^r	Invitrogen
pGRV2	Gene replacement suicide vector, Gm^r	63
pDD159	*sacB* gene deletion allele	This study
pCRXL1517SOE	Spliced flanking regions of BMAA1517	This study
pCRXL1621SOE	Spliced flanking regions of BMAA1621	This study
pGRV1517SOE	BamHI-XbaI fragment from pCRXL1517SOE	This study
pGRV1621SOE	SacI-BamHI fragment from pCRXL1621SOE	This study

[a] Pm, polymyxin B; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Zeo, zeocin; Amp, ampicillin.
[b] A gene was inactivated by insertion of a pCR-XL-TOPO vector containing a 400-to 900-bp PCR-amplified internal fragment of the gene.

(T6S) gene cluster was shown to be required for the virulence of *B. mallei* (51, 56). Several putative virulence factors, including two proteases (10, 41), a hemolysin (1), a rhamnolipid (28), predicted transporter proteins, and three hypothetical proteins, have been identified using either nematode killing assays (24) or biochemical analysis. However, these factors are yet to be verified by animal experiments. Recently, Tuanyok et al. (62) identified a phospholipase C and a two-component regulator which are required for full virulence of *B. pseudomallei*.

The genome sequences of >25 *Burkholderiaceae* isolates representing >12 species and four genera are available (http://www.genomesonline.org/). These isolates occupy a wide diversity of ecological niches, yet they share a common core genome comprised of long syntenic regions containing >2,500 shared "housekeeping" genes whose products share >60% amino acid sequence identity. Of greater interest are the species- or isolate-specific genes, such as those found only in human-pathogenic *Burkholderia* spp. Therefore, we used in silico genomic subtraction (22) to derive a set of 650 genes that are present in both *B. pseudomallei* and *B. mallei* but are not present in the sequenced nonpathogenic members of the *Burkholderiaceae*. This set is predicted to be highly enriched in novel virulence genes and has been designated the Bm-Bp virulome. We investigated this virulome by selecting genes likely to encode novel virulence factors, inactivating them, and testing the corresponding mutants to determine whether they exhibited reduced virulence in wax moth larvae, a surrogate model that we adapted and verified as correlating with the hamster model commonly used to test *B. mallei* virulence. Our results show that this is a powerful and easily applicable approach that can accelerate research on these important pathogens and serve as an adjunct to much more expensive and problematic animal testing.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and plasmids used are listed in Table 1. Bacteria were grown at 37°C in LB (45) medium supplemented with 2% glycerol (LBG) or in brain heart infusion (BHI) medium. For some experiments, *B. mallei* was also grown on minimal medium plates (4) with 5% sucrose as the sole carbon source. When necessary, antibiotics were used at the following concentrations: kanamycin, 50 μg/ml; polymyxin B, 20 μg/ml; and gentamicin, 20 μg/ml.

Preparation of electrocompetent *B. mallei*. An overnight culture was diluted to obtain an A_{600} of 0.1 in BHI medium and shaken for ~3 h until the A_{600} was 0.8. Cells were harvested by centrifugation at 6°C at 4,000 × *g* and washed with 0.5 culture volume of ice-cold sterile water and then with 0.25 volume of ice-cold sterile 10% glycerol. Cells were finally resuspended in 0.01 volume of 10% glycerol and frozen at −80°C.

Construction of a *sacB* deletion strain of *B. mallei* ATCC 23344. Four PCR primers (see Table S1 in the supplemental material) were used to generate DNA fragments of the 5′ and 3′ ends of the *sacB* gene (BMAA0466) from *B. mallei* ATCC 23344, and the fragments were cloned into separate pCR2.1-TOPO vectors. The Sac7/Sac8 product was cut out of pCR2.1-TOPO with EcoRI, and the Sac5/Sac6 product was cut out with XbaI and BamHI. Both fragments were cloned stepwise into the corresponding sites of pGRV2 (63), resulting in

pDD159, which was confirmed by sequencing. Subsequently, pDD159 was transformed into *Escherichia coli* S17-1 (56) and mobilized into *B. mallei* ATCC 23344. Transconjugants were inoculated into LBG broth without antibiotics, and 10-fold dilutions of the overnight cultures were spread onto LBG plates with 5% sucrose. Sucrose-resistant colonies were screened for the *sacB* deletion mutation by PCR, and one confirmed isolate was designated *B. mallei* GRS 23344.

Construction of putative virulence mutant. Internal fragments (400 to 900 bp; lacking both 5′ and 3′ ends) of target coding sequences (CDSs) were PCR amplified with appropriate primers (see Table S1 in the supplemental material), directly cloned into pCR-XL-TOPO (Invitrogen), and transformed into chemically competent *E. coli* TOP10. Approximately 0.5 μg of each of the resultant plasmids was electroporated into 70 μl of ice-cold electrocompetent *B. mallei* cells with an Eppendorf w-150 electroporator at 2.3 kV, 600 Ω, and 10 μF for 5 ms. Cells were immediately diluted 10-fold with LBG and shaken for 4 h at 37°C, after which aliquots were spread onto LBG plates containing kanamycin. Colonies that grew after 3 to 4 days were picked, the genomic DNA was isolated, and the presence of the appropriate *cis*-merodiploid mutant allele was verified by PCR. Typically, 25% of the colonies harbored the expected mutant allele.

BMAA1517 and BMAA1621 were refractory to insertional inactivation, so we used splice overlap extension PCR (32) with a modified *sacB*-based deletion method (43, 63). Briefly, four PCR primers (see Table S1 in the supplemental material) were used to amplify two ~500-bp fragments of DNA flanking each target gene. Gel-purified amplicons were mixed in a second PCR mixture with the forward primer of the upstream amplicon and the reverse primer of the downstream amplicon. The ~1,000-bp chimeric fragments were cloned into pCR-XL-TOPO, cut out with restriction enzymes (Table 1), and ligated into the appropriate sites in pGRV2 (63). The resulting plasmids were transformed into *E. coli* S17-1 (57) and mobilized into the *B. mallei sacB* deletion mutant GRS 23344. Transconjugants were spread onto minimal medium sucrose plates. Colonies of putative deletion mutants that grew after 5 to 7 days of incubation were screened by PCR to verify deletion of the target sequence. Approximately 10% of the colonies tested harbored the deletion allele.

Infection of *Galleria mellonella* larvae. *B. mallei* strains were grown at 37°C for 24 to 48 h on LBG plates, resuspended at an A_{600} of 0.2 in BHI medium, and shaken at 37°C until the A_{600} was between 0.4 and 0.6. Culture aliquots were diluted in sterile water, and ~10 or 250 cells in 5 μl were injected under the carapace into the hemocoel of 10 fifth-instar *G. mellonella* larvae (Vanderhorst Wholesale, Marysville OH) using a 50-μl gas-tight Hamilton syringe equipped with a 30.5-gauge needle (Becton Dickinson 305106). Inoculated larvae were sealed in 2-oz hinged-lid polypropylene cups (Fisher 03-405-39) with five 2-mm ventilation holes in the lid. Larval death was scored daily for 1 week; dead larvae no longer moved when the cup was tapped and/or had turned black.

Bioinformatic methods. In silico genomic subtraction was performed as previously described (22). Briefly, using BLASTP, the amino acid sequences encoded by all ~6,000 Glimmer-predicted CDSs of *B. mallei* ATCC 23344 were individually aligned with the best hits for predicted CDSs of five closely related nonpathogenic strains: *Burkholderia* sp. strain 383 (= ATCC 17660 = R-18194), *Ralstonia eutropha* JMP134, *Burkholderia ambifaria* AMMD, *Burkholderia xenovorans* LB400, and *Burkholderia vietnamiensis* G4. The *Burkholderia* strains were chosen because they are nonclinical (environmental) isolates with a long history of use and no reports of human infection. Sequences of their CDS products and contigs were obtained from JGI/ORNL (http://compbio.ornl.gov/channel). For each CDS alignment, an "orthology score" was calculated by using amino acid sequence identity, the ratio of alignment and query lengths, and the ratio of query and hit lengths. Utilizing an orthology score cutoff value based on the average amino acid sequence identities of the 100 most similar ortholog pairs of each, a set of *B. mallei* CDS products lacking orthologs in any of the closely related nonpathogens was derived. TBLASTN was then used to compare each CDS in this set to the genomic DNA sequence of the same five bacteria to find and remove orthologous CDSs of *B. mallei* present in the DNA sequence but not in the called CDS sets. This method provided results similar to those obtained with the reciprocal best BLAST hit method but, in contrast, can be used with raw unfinished genome sequences (22).

Select agent handling. Gm^r *B. mallei* strains constructed during this work were generated before the Centers for Disease Control Select Agent Program (CDC-SAP) informed us that, despite the fact that Gm^r had been widely used in *B. mallei* before 2006, introduction of Gm^r into *B. mallei* is now considered a "restricted experiment" that requires prior CDC-SAP approval. Thus, all Gm^r *B. mallei* strains that we constructed have been destroyed. Otherwise, all regulations and procedures mandated by CDC-SAP were followed during this work.

RESULTS AND DISCUSSION

Derivation of a virulome for *B. mallei* and *B. pseudomallei*. Despite the extensive ecological diversity of the organisms, genome comparisons of members of the *Burkholderiaceae* have revealed a core genome comprised of long syntenic regions containing thousands of shared "housekeeping" genes. Of greater interest are the hundreds of species- or isolate-specific genes that confer on each isolate its ability to colonize and survive in a specific niche or host. To find genes that are specific to *B. mallei* and *B. pseudomallei* and also have increased probability of major involvement in virulence, we used an in silico genomic subtraction method in which the amino acid sequences encoded by all *B. mallei* CDSs were individually compared to the amino acid sequences encoded by all the CDSs of four closely related nonpathogenic *Burkholderia* strains, and all CDS products found in "orthologous" pairs were removed. This yielded a set of 650 CDSs designated the Bm-Bp virulome (see Table S2 in the supplemental material), all of which are present in both *B. mallei* and *B. pseudomallei* but whose products have <45% amino acid sequence identity with the product of any other CDS in the genomes of the nonpathogens. In contrast, the average amino acid identity observed for most orthologous CDS pairs is >65%. This virulome comprises ~12% of the genome of *B. mallei* and appears to be highly enriched in CDSs/genes encoding probable virulence and pathogenicity factors since it contains most of the genes in the animal pathogen-like T3SS ($T3SS_{AP}$), CAP, T6S, and LPS biosynthetic gene clusters, the only published loci experimentally proven to be critical for causing disease in the hamster model (14, 56, 64). When we attempted to use the total CDSs of *Burkholderia thailandensis* E264, a strain previously considered a nonpathogen (26, 47), in a subsequent in silico subtraction experiment to further reduce and refine the virulome, we found orthologs for 70% of the virulome CDSs in the nonpathogen, including the majority of the CDSs in the three large gene clusters (T3SS, T6S, and CAP) that are essential for *B. mallei* virulence. In contrast, orthologs for <10% of the virulome CDSs were found in the genome of *Burkholderia cenocepacia* J2315 or *B. cenocepacia* AU1054, two pathogenic strains isolated from cystic fibrosis patients (5, 44, 65). This is perhaps not surprising considering that *B. thailandensis* E264 is very closely related to *B. pseudomallei* and that at relatively high doses it has the ability to kill nematodes (50) and hamsters (2) and has the ability to survive in macrophages (F. Gherardini, personal communication).

Most of the 650 virulome CDSs occur in ~40 operonlike clusters of four or more contiguous genes (only 100 genes are singletons). Not surprisingly, most clusters are on the smaller replicon, which carries the majority of nonhousekeeping genes (31, 49). Particularly high concentrations of the CDSs are found between BMAA0345 and BMAA0410, between BMAA0729 and BMAA 0758, and between BMAA1517 and BMAA1565. Very few of the CDSs in clusters have Karlin score deviations (38), G+C contents, or GC skews that significantly differ from those of the flanking regions or the genome averages. Nearly 130 CDSs are predicted by SignalP V.3.0 (48) to encode signal peptides for secretion, a value which is about four times higher than the predicted genome-wide average. In the clusters with >10 genes are two complete sets of T3SS genes and several predicted secondary

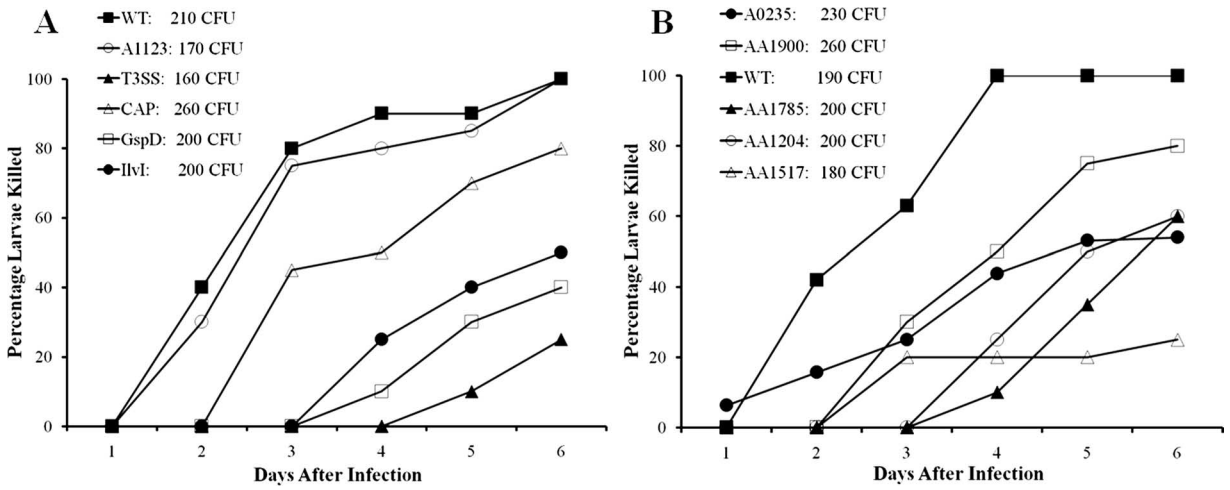

FIG. 1. Killing curves for *G. mellonella* larvae inoculated with wild-type and mutant *B. mallei* cells. (A) Time course of larval killing by *B. mallei* wild type (WT) and mutants with defects in characterized virulence genes. (B) Time course of larval killing by wild type and mutants with defects in genes selected from the Bm-Bp virulome. Assays were performed at least three independent times by inoculating 10 larvae with each mutant. The actual number of cells (CFU) injected into the larvae was determined by plating duplicate aliquots of the injectant on LBG plates. The percentages of larvae killed are the averages of three assays; the standard deviations are <15%. Wild-type *B. mallei* and water-inoculated controls were included with each set of assays.

metabolite-producing clusters encoding nonribosomal peptide or polyketide synthetases possibly involved in the cytotoxic activities ascribed to *B. pseudomallei* (6, 29, 30). Also present are CDSs encoding major parts of both the CAP and LPS biosynthesis clusters and two fimbria/pilus biogenesis pathways, in addition to >100 CDSs encoding predicted membrane proteins. The presence of CDSs encoding proteins with predicted enzymatic functions in other clusters suggests that these CDSs may encode parts of metabolic pathways. However, many of the clustered genes have no predicted function; >200 CDSs lack any functional description, and ~100 do not have PFAM domains.

***G. mellonella* larvae are a valid surrogate host for *B. mallei*.** The ultimate goal of deriving the virulome was to extract a comprehensive set of potentially novel virulence genes that contained few enough genes that the majority could be individually knocked out and mutants evaluated to determine whether the knockouts reduced virulence in animal models. Uncertainty about the pathogenic potential of *B. thailandensis* precluded using in silico subtraction to reduce the virulome to a size at which we could realistically test a large proportion of the genes in mammalian models under cumbersome BSL3 conditions. In contrast, higher-throughput surrogate host systems can be very simple and inexpensive, yet quite accurate for study of human pathogens (61). *G. mellonella* (wax moth) larvae have served as an insect host and simple pathogenesis model for several mammalian pathogens (39); they are easy to acquire and contain without special facilities. Most importantly, *G. mellonella* has a circulatory system and a complex innate and mammal-like immune response that effectively deals with a wide range of microbial pathogens (39). In some cases the virulence of a pathogen and attenuated mutants in animal hosts has been accurately reflected in *G. mellonella* (33, 46). Therefore, we tested *G. mellonella* as a pathogenesis model for verification of *B. mallei* virulence genes in the Bm-Bp virulome.

When ~10 wild-type *B. mallei* ATCC 23344 (or *B. mallei* GRS 23344) cells were injected into the hemocoel of larvae, the majority of the larvae were killed within 4 days. In ~20 experiments we inoculated more than 300 larvae with anywhere from 3 to 200 cells of wild-type *B. mallei* and always observed >90% killing within 6 days. Injecting 10 *B. pseudomallei* K96243 cells into the larvae killed them nearly twice as fast (>80% mortality by 2 days). Similar to hamsters infected with *B. mallei* or *B. pseudomallei*, infected larvae showed extensive paralysis 12 h before death. At the onset of paralysis, typically $>10^4$ *B. mallei* cells/ml were present in the hemolymph; just prior to death $>10^6$ *B. mallei* or *B. pseudomallei* cells/ml were found. These results indicate that *B. mallei* and *B. pseudomallei* can multiply to very high numbers in the hemocoel of wax moth larvae and are highly pathogenic for this insect. Injection of up to 10^5 cells of the pathogenic cystic fibrosis epidemic strain *B. cenocepacia* J2315, *Pseudomonas putida*, *B. xenovorans*, or *E. coli* W3110 killed <15% of the larvae by day 6; using an inoculum containing 10^4 cells reduced the percentage of dead larvae at day 6 to <10%, which is identical to the level observed if water was injected. Injection of $>10^5$ *B. thailandensis* cells killed ~35% of the larvae after 7 days. Although this is somewhat inconsistent with the "nonpathogenic" label for *B. thailandensis*, as mentioned above, this species harbors ~70% of the genes in the Bm-Bp virulome and at relatively high doses ($>10^5$ cells) it also can kill hamsters and nematodes. Moreover, at least one opportunistic infection of a human by *B. thailandensis* has been reported (26).

To show correspondence between the wax moth larva model and mammalian models, we first tested *B. mallei* RD01, which has a polar insertion in the $T3SS_{AP}$ gene cluster (64). This T3SS is essential for the virulence of *B. mallei* and *B. pseudomallei* in mice and hamsters, respectively (64, 66). Compared to the wild type, the ability of *B. mallei* RD01 to kill wax moth larvae was dramatically reduced (Fig. 1A, compare WT and T3SS); at lower infection levels (<20 cells/larva), RD01 was largely avirulent (data not shown). When *B. mallei* strain

TABLE 2. Potential virulence genes targeted for mutagenesis

B. mallei CDS	Product[a]	Rationale for targeting	Virulence of mutant in larvae[b]
BMA0235	3-Phosphoshikimate 1-carboxyvinyltransferase	Best BLASTP hit in phylogenetically distant pathogen	Reduced
BMA0847	Galactose oxidase-related protein	Best BLASTP hit in eukaryote	Normal
BMA1123	Peptide synthetase	Possible role in cytotoxin production	Normal
BMAA1013	ECF sigma factor	Potential virulence gene regulator	Normal
BMAA1111	Hypothetical protein	Best BLASTP hit in eukaryote	Normal
BMAA1204	Putative PKS	Possible role in cytotoxin production	Reduced
BMAA1517	AraC family transcriptional regulator	Potential virulence gene regulator	Reduced
BMAA1621	Regulatory protein HrpB	Potential virulence gene regulator	Normal
BMAA1785	Chitin binding domain protein	Best BLASTP hit in phylogenetically distant pathogen	Reduced
BMAA1900	Pentapeptide repeat family protein	Best BLASTP hit in phylogenetically distant pathogen	Reduced

[a] Gene product description in Integrated Microbial Genomes (http://img.jgi.doe.gov/).
[b] See text and Fig. 1B.

DD3008, a CPS-deficient mutant with dramatically reduced virulence in hamsters (10), was used, the killing of wax moth larvae was delayed and reduced (Fig. 1A, compare WT and CAP). Next, we constructed an isoleucine-valine auxotroph of *B. mallei* by insertional inactivation of *ilvI* (BMA1848) encoding a subunit of acetolactate synthase III. The resultant mutant was not able to grow on minimal glucose medium and, similar to what has been observed with mice (63), showed attenuated virulence in larvae (Fig. 1A, compare WT and IlvI). Thus, the behavior of the three mutants whose virulence in animal models is attenuated correlated well with their virulence in larvae. Inactivation of type II secretion affects the virulence of *P. aeruginosa* (17, 69), some plant pathogens (36, 43), and *B. pseudomallei* (10). Therefore, we insertionally inactivated *gspD* (BMA2786), a key component of type II secretion, and found that the killing of wax moth larvae by the resultant GspD mutant was reduced and delayed (Fig. 1A), but not to the extent caused by the T3SS mutation. These data suggest that the wax moth model of infection is an efficient predictor of potential virulence genes in *B. mallei*.

To verify the stability of the mutant genotype in the wax moth larvae, bacterial cells were recovered from the hemolymph at days 2 and 5 by plating on nonselective LBG agar. All of the 250 resultant colonies tested retained the antibiotic resistance encoded by the plasmid insertion. When auxotrophic *ilvI* mutant cells were recovered from larval hemolymph and plated on both LBG and minimal media, we found that <1 in 10^5 of the ex vivo cells had reverted to prototrophy, further demonstrating the high level of stability of the *cis*-merodiploid mutants.

Use of the wax moth larva model to screen putative virulence mutants. Based on a manual review of bioinformatic data for virulome CDSs, 10 CDSs were evaluated to determine whether they have a role in virulence (Table 2). Some of these CDSs were chosen because their best BLAST hits were in phylogenetically distant pathogens or in a eukaryote, some were chosen because they appeared to be potential virulence gene regulators, and some were chosen because they appeared to encode polyketide or peptide synthetases that may be involved in cytotoxin production. Knockout mutants for each CDS were constructed and verified by PCR, and their virulence in wax moth larvae was tested as described above. *B. mallei* strains with mutations in BMA0847, BMAA1111, BMA1123, BMAA1013, and BMAA1621 had marginal or no differences in virulence compared with the wild type (Fig. 1A and data not shown), whereas with strains harboring insertions in BMA0235, BMAA1204, BMAA1517, BMAA1785, and BMAA1900 there was reduced and/or delayed killing of the larvae (Fig. 1B).

Of all the mutants tested, inactivation of BMAA1517, encoding an AraC-type transcriptional regulator, resulted in the most dramatic reduction in virulence in the larvae. At 7 days postinfection 80% of the larvae infected with ~200 CFU of the BMAA1517 mutant remained asymptomatic, similar to the behavior of the $T3SS_{AP}$ mutant. Whole-genome expression profiling of a *B. mallei* strain overexpressing BMAA1517 has shown that a cluster of genes found in the Bm-Bp virulome (BMAA0727 to BMAA0744) is exclusively upexpressed on average 4-fold; the levels of some genes are elevated >10-fold (56). This previous study also showed that these genes encode components of a T6S system which, when inactivated, rendered *B. mallei* avirulent in hamsters. When we tested a mutant lacking a critical component of the T6S system (BMAA0739), its virulence in larvae was reduced to the same extent as that of BMAA1517 mutants (not shown). These results further support the hypothesis that the wax moth larva model is an accurate predictor of *B. mallei* virulence genes.

Inactivation of BMA0235 in *B. mallei* dramatically delayed and reduced killing of larvae in the wax moth infection model. BMA0235 is in an operonlike cluster of six genes that is conserved in all sequenced *B. pseudomallei* and *B. mallei* strains but is not present in any other *Burkholderia* genome or in the 600 microbial genomes at Integrated Microbial Genomes (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi), with the exception of the genome of *Photorhabdus luminescens*. In fact, *P. luminescens*, which can be an insect pathogen or nematode symbiont (21), has similarly organized orthologs of the five genes flanking BMA0235 (Fig. 2). The average amino acid identity between the products of the six CDS pairs from these phylogenetically distant bacteria is 66%, which is quite remarkable considering that the G+C content of the *P. luminescens* CDSs is ~38%, while the average G+C content of the *B. mallei* orthologs is 68%. The BMA0237 protein has a domain

CDS	% ID	Predicted Function
BMA237/PLU2767	76	Mutase
BMA236a/PLU2768	50	Carboxyvinyl transferase A
BMA235/PLU2769	72	Carboxyvinyl transferase B
BMA234/PLU2770	70	Amidotransferase
BMA233a/PLU2766	53	Carboxylase COG0439
BMA232/PLU2765	74	DMT metabolite transporter COG0697

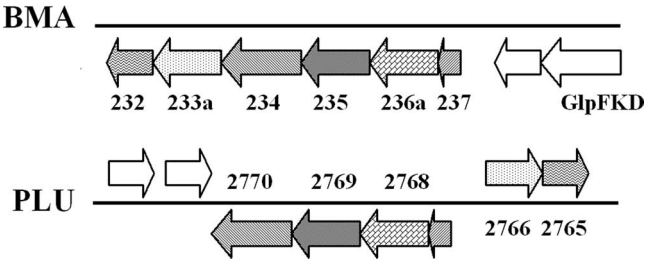

FIG. 2. Organization and amino acid sequence identity for orthologous genes in *B. mallei* (BMA) and *P. luminescens* (PLU) that may encode an insect toxin. Orthologous genes have the same arrow pattern. The location of the *B. mallei* CDS (BMA0235) that was disrupted by insertion is indicated. DMT, drug/metabolite transporter.

similar to a domain in chorismate mutase (PFAM1042), while the BMA0236a and BMA0235 proteins are both assigned to the enoylpyruvate carboxytransferase family (PFAM 00275) and are related to 3-phosphoshikimate 1-carboxyvinyl transferases. Both of the latter proteins were originally annotated as AroA (EC 2.5.1.19), which is involved in aromatic amino acid biosynthesis. However, these proteins show <25% identity to each other and to *E. coli* AroA. The AroA function of *B. mallei* more likely resides in the BMA0430 protein, annotated as a bifunctional prephenate dehydrogenase/3-phosphoshikimate 1-carboxyvinyl transferase enzyme whose C-terminal and N-terminal halves show ~50% amino acid identity to full-length *E. coli* AroA and TyrA, respectively. Thus, the BMA0235 and BMA0236a proteins are more likely novel carboxyvinyl transferases that transfer a vinyl group from phosphoenolpyruvate to an unknown substrate. The BMA0234 protein is annotated as a putative asparagine synthase B (EC 6.3.5.4), as are the products of two other CDSs in the *B. mallei* genome (BMAA1158 and BMAA1921). The products of these three CDSs do not show >30% amino acid identity to *E. coli* AsnB. The BMA0234 protein is likely involved in an AsnB-like reaction, transfer of the amido group of glutamine to an aspartate-like substrate. The BMA0233a protein may be a carboxylase, while the BMA0232 protein is a putative drug/metabolite transporter (PFAM 05297). It is plausible that the gene clusters shown in Fig. 2 synthesize a secondary metabolite or toxin that plays a role in insect pathogenesis in *P. luminescens* and perhaps in *B. mallei*. This metabolite could be derived from carboxyvinylation of 3,4-dihydroxy-2-butanone 4-phosphate made by the adjacent *ribA* gene, followed by amidation of the carboxyvinyl group. *P. luminescens* and its close relatives are well known for production of insect toxins.

BMAA1204 mutants took twice as long as the wild type to start killing larvae; after 7 days only 60% of the mutant-infected larvae were dead, compared to the 100% mortality caused by the wild type. BMAA1204 is a ~4,200-residue CDS annotated as encoding a putative polyketide synthase (PKS) in COG family 0332. PKSs are giant multidomain proteins ubiquitous in the prokaryotes. In a manner analogous to fatty acid biosynthesis, they condense or "polymerize" organic acids (e.g., acetate and malonate) into linear polyketides. The resultant polyketides are often cyclized and modified, yielding 300- to 1,500-Da molecules that exhibit diverse antibiotic, antitumor, or even immunosuppressive activities. BMAA1204 is in a large gene cluster along with genes encoding two other putative PKSs and several CDSs whose products are in protein families associated with polyketide synthesis and/or modification. In fact, most CDSs in this region (BMAA1201 to BMAA1222) are in the virulome. It is possible that BMAA 1204 and/or the adjacent PKS genes encode production of one of the "cytotoxin" activities found previously in culture supernatants of *B. pseudomallei* (16, 41, 42), whose characteristics (e.g., heat stability and molecular weight) are consistent with a polyketide origin. PKSs have been implicated in pathogenesis by several other bacteria (e.g., *Mycobacterium ulcerans* [35]). *B. mallei* has seven putative PKS genes; three of these genes (BMAA1021, BMAA1204, and BMAA1451) are present in the virulome but are not present in *B. thailandensis*.

Inactivation of BMAA1785, whose product is annotated as a "chitin binding domain protein," reduced virulence quantitatively to an extent very similar to the extent observed after inactivation of the PKS gene BMAA1204. Although nonpathogenic *Burkholderia* spp. lack orthologs of the BMAA1785 protein, *Yersinia pestis* and *Yersinia pseudotuberculosis* have orthologs with 55% amino acid sequence identity. The actual function of the BMAA1785 protein is not clear, but its so-called chitin binding domain 3 (PFAM03067) is related to baculoviral spheroidin and spindolin capsid proteins. It has a predicted signal sequence and is present at moderately high levels in culture supernatants of *B. pseudomallei* and *B. mallei* (52; data not shown). We speculate that it binds to some type of chitin-like oligosaccharide moiety and has an as-yet-unknown function.

Inactivation of BMAA1900 caused only a moderate delay (1 day) in the onset of killing and a minor reduction in *B. mallei*-related mortality of wax moth larvae. This gene is in a cluster (BMAA1897 to BMAA1915) that is predicted to encode one of the four predicted T6S systems in *B. mallei* (56). BMAA1900 encodes a putative pentapeptide repeat protein (COG family 1357) that is 38% identical to a putative exported protein of *Bordetella bronchiseptica*, which is located in an analogous T6S system cluster. While one can speculate that BMAA1900 is involved in T6S system-mediated secretion of a virulence factor(s), it seems that this T6S system plays a relatively minor role in virulence for wax moth larvae.

Conclusion. To date, relatively few virulence factors of *B. mallei* have been identified, and most of the factors that have been found are widespread in diverse pathogens (e.g., T3SS and capsule). Some of the methods used to identify these virulence factors, such as subtractive hybridization and transposon mutagenesis, are time-consuming and especially cumbersome with BSL3-requiring select agent pathogens. With the availability of hundreds of bacterial genome sequences, it makes sense to exploit these databases to use new bioinformatic methods to direct functional genomic studies. In silico genomic subtraction provided a set of *B. mallei* genes that contained genes encoding all known virulence factors and

therefore was very valuable as a starting point for functional analyses to discover new genes with a role in pathogenesis.

G. mellonella larvae have successfully been used as a model for infection by several pathogenic bacteria (39), as well as in screening for bacterial mutants with reduced virulence and/or altered host colonization ability (33, 46). We have shown that *G. mellonella* also is a good model for *B. mallei* pathogenesis, because four *B. mallei* strains with mutations in different characterized virulence gene clusters (T3SS, CAP, type II secretion system, and T6S system) showed reduced virulence that correlated well with their behavior in the hamster model of infection. The larva model is a facile and inexpensive method that can be used to test potential virulence mutants of *B. mallei* before hamster or mouse models are used. The value of the larva model is enhanced by the observation that *G. mellonella* has many sophisticated defenses analogous to those found in mammals, such as circulating hemocytes utilizing surface receptors similar to those found in mammalian phagocytes to help encapsulate and destroy microbial invaders (39). Other antimicrobial responses of these insects to pathogens are also similar to those of mammals, including oxidative burst and inducible production of lysozyme and other antibacterial proteins, such as cecropins, defensins, and proline-rich peptides. Besides demonstrating the utility and validity of the *G. mellonella* insect model for screening mutants for virulence phenotypes, some of our results (i.e., the killing of larvae by very low infectious doses and the presence of multiple insect pathogen-associated genes in the virulome [Fig. 2; see Table S2 in the supplemental material]) suggest that some soil-dwelling insects may be natural hosts and reservoirs of *B. pseudomallei* (and perhaps *B. mallei*). However, if this is true, the use of the larva model may result in a bias toward discovery of genes for virulence in insects that may or may not play as significant a role in mammalian pathogenesis.

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REFERENCES

1. **Ashdown, L. R., and J. M. Koehler.** 1990. Production of hemolysin and other extracellular enzymes by clinical isolates of *Pseudomonas pseudomallei*. J. Clin. Microbiol. **28:**2331–2334.
2. **Brook, M. D., B. Currie, and P. M. Desmarchelier.** 1997. Isolation and identification of *Burkholderia pseudomallei* from soil using selective culture techniques and the polymerase chain reaction. J. Appl. Microbiol. **82:**589–596.
3. **Christopher, G. W., T. J. Cieslak, J. A. Pavlin, and E. M. Eitzen, Jr.** 1997. Biological warfare. A historical perspective. JAMA **278:**412–417.
4. **Clough, S. J., M. A. Schell, and T. P. Denny.** 1994. Evidence for involvement of a volatile extracellular factor in *Pseudomonas solanacearum* virulence gene expression. Mol. Plant-Microbe Interact. **7:**621–630.
5. **Coenye, T., T. Spilker, A. Van Schoor, J. J. LiPuma, and P. Vandamme.** 2004. Recovery of *Burkholderia cenocepacia* strain PHDC from cystic fibrosis patients in Europe. Thorax **59:**952–954.
6. **Colling, M., C. Nigg, and R. J. Heckly.** 1958. Toxins of *Pseudomonas pseudomallei*. I. Production in vitro. J. Bacteriol. **76:**422–426.
7. **Currie, B. J., D. A. Fisher, D. M. Howard, J. N. Burrow, D. Lo, S. Selva-Nayagam, N. M. Anstey, S. E. Huffam, P. L. Snelling, P. J. Marks, D. P. Stephens, G. D. Lum, S. P. Jacups, and V. L. Krause.** 2000. Endemic melioidosis in tropical northern Australia: a 10-year prospective study and review of the literature. Clin. Infect. Dis. **31:**981–986.
8. **Dance, D. A.** 2000. Ecology of *Burkholderia pseudomallei* and the interactions between environmental *Burkholderia* spp. and human-animal hosts. Acta Trop. **74:**159–168.
9. **Dance, D. A.** 2002. Melioidosis. Curr. Opin. Infect. Dis. **15:**127–132.
10. **DeShazer, D., P. J. Brett, M. N. Burtnick, and D. E. Woods.** 1999. Molecular characterization of genetic loci required for secretion of exoproducts in *Burkholderia pseudomallei*. J. Bacteriol. **181:**4661–4664.
11. **DeShazer, D., P. J. Brett, R. Carlyon, and D. E. Woods.** 1997. Mutagenesis of *Burkholderia pseudomallei* with Tn5-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. J. Bacteriol. **179:**2116–2125.
12. **DeShazer, D., P. J. Brett, and D. E. Woods.** 1998. The type II O-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. Mol. Microbiol. **30:**1081–1100.
13. **DeShazer, D., and D. M. Waag.** 2004. Glanders: new insights into an old disease, p. 209–237. *In* L. Lindler, F. Lebada, and G. W. Korch (ed.), Biological weapons defense: principles and mechanisms for infectious diseases counter-bioterrorism. Humana Press, Inc., Totowa, NJ.
14. **DeShazer, D., D. M. Waag, D. L. Fritz, and D. E. Woods.** 2001. Identification of a *Burkholderia mallei* polysaccharide gene cluster by subtractive hybridization and demonstration that the encoded capsule is an essential virulence determinant. Microb. Pathog. **30:**253–269.
15. **DeShazer, D., and D. Woods.** 1999. Animal models of melioidosis, p. 199–203. *In* O. Zak and M. Sande (ed.), Handbook of animal models of infection. Academic Press Ltd., London, United Kingdom.
16. **Dharakul, T., and S. Songsivilai.** 1999. The many facets of melioidosis. Trends Microbiol. **7:**138–140.
17. **Durand, E., A. Bernadac, G. Ball, A. Lazdunski, J. N. Sturgis, and A. Filloux.** 2003. Type II protein secretion in *Pseudomonas aeruginosa*: the pseudopilus is a multifibrillar and adhesive structure. J. Bacteriol. **185:**2749–2758.
18. **Essex-Lopresti, A. E., J. A. Boddey, R. Thomas, M. P. Smith, M. G. Hartley, T. Atkins, N. F. Brown, C. H. Tsang, I. R. A. Peak, and J. Hill.** 2004. A type IV pilin, PilA, contributes to adherence of *Burkholderia pseudomallei* and virulence in vivo Infect. Immun. **73:**1260–1264.
19. **Finlay, B. B., and P. Cossart.** 1997. Exploitation of mammalian host cell functions by bacterial pathogens. Science **276:**718–725.
20. **Finlay, B. B., and S. Falkow.** 1997. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. **61:**136–169.
21. **Fischer-Le Saux, M., V. Viallard, B. Brunel, P. Normand, and N. E. Boemare.** 1999. Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens* subsp. nov., *P. luminescens* subsp. *akhurstii* subsp. nov., *P. luminescens* subsp. *laumondii* subsp. nov., *P. temperata* sp. nov., *P. temperata* subsp. *temperata* subsp. nov., and *P. asymbiotica* sp. nov. Int. J. Syst. Bacteriol. **49:**1645–1656.
22. **Gabriel, D. W., C. Allen, M. Schell, T. P. Denny, J. T. Greenberg, Y. P. Duan, Z. Flores-Cruz, Q. Huang, J. M. Clifford, G. Presting, E. T. Gonzalez, J. Reddy, J. Elphinstone, J. Swanson, J. Yao, V. Mulholland, L. Liu, W. Farmerie, M. Patnaikuni, B. Balogh, D. Norman, A. Alvarez, J. A. Castillo, J. Jones, G. Saddler, T. Walunas, A. Zhukov, and N. Mikhailova.** 2006. Identification of open reading frames unique to a select agent: *Ralstonia solanacearum* race 3 biovar 2. Mol. Plant-Microbe Interact. **19:**69–79.
23. **Galan, J. E., and A. Collmer.** 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. Science **284:**1322–1328.
24. **Gan, Y. H., K. L. Chua, H. H. Chua, B. Liu, C. S. Hii, H. L. Chong, and P. Tan.** 2002. Characterization of *Burkholderia pseudomallei* infection and identification of novel virulence factors using a *Caenorhabditis elegans* host system. Mol. Microbiol. **44:**1185–1197.
25. **Gauthier, Y. P., R. M. Hagen, G. S. Brochier, H. Neubauer, W. D. Splettstoesser, E. J. Finke, and D. R. Vidal.** 2001. Study on the pathophysiology of experimental *Burkholderia pseudomallei* infection in mice. FEMS Immunol. Med. Microbiol. **30:**53–63.
26. **Glass, M. B., J. E. Gee, A. G. Steigerwalt, D. Cavuoti, T. Barton, R. D. Hardy, D. Godoy, B. G. Spratt, T. A. Clark, and P. P. Wilkins.** 2006. Pneumonia and septicemia caused by *Burkholderia thailandensis* in the United States. J. Clin. Microbiol. **44:**4601–4604.
27. **Godoy, D., G. Randle, A. J. Simpson, D. M. Aanensen, T. L. Pitt, R. Kinoshita, and B. G. Spratt.** 2003. Multilocus sequence typing and evolutionary relationships among the causative agents of melioidosis and glanders, *Burkholderia pseudomallei* and *Burkholderia mallei*. J. Clin. Microbiol. **41:**2068–2079.
28. **Haussler, S., M. Nimtz, T. Domke, V. Wray, and I. Steinmetz.** 1998. Purification and characterization of a cytotoxic exolipid of *Burkholderia pseudomallei*. Infect. Immun. **66:**1588–1593.
29. **Heckly, R. J.** 1964. Differentiation of exotoxin and other biologically active substances in *Pseudomonas pseudomallei* filtrates. J. Bacteriol. **88:**1730–1736.
30. **Heckly, R. J., and C. Nigg.** 1958. Toxins of *Pseudomonas pseudomallei*. II. Characterization. J. Bacteriol. **76:**427–436.
31. **Holden, M. T., R. W. Titball, S. J. Peacock, A. M. Cerdeno-Tarraga, T. Atkins, L. C. Crossman, T. Pitt, C. Churcher, K. Mungall, S. D. Bentley, M. Sebaihia, N. R. Thomson, N. Bason, I. R. Beacham, K. Brooks, K. A. Brown, N. F. Brown, G. L. Challis, I. Cherevach, T. Chillingworth, A. Cronin, B. Crossett, P. Davis, D. DeShazer, T. Feltwell, A. Fraser, Z. Hance, H. Hauser, S. Holroyd, K. Jagels, K. E. Keith, M. Maddison, S. Moule, C. Price, M. A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, M. Simmonds, S. Songsivilai, K. Stevens, S. Tumapa, M. Vesaratchavest, S. Whitehead, C.**

Yeats, B. G. Barrell, P. C. Oyston, and J. Parkhill. 2004. Genomic plasticity of the causative agent of melioidosis, Burkholderia pseudomallei. Proc. Natl. Acad. Sci. USA **101:**14240–14245.
32. **Horton, R. M., S. N. Ho, J. K. Pullen, H. D. Hunt, Z. L. Cai, and L. R. Pease.** 1993. Gene-splicing by overlap extension. Methods Enzymol. **217:**270–279.
33. **Jander, G., L. G. Rahme, and F. M. Ausubel.** 2000. Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. J. Bacteriol. **182:**3843–3845.
34. **Jeddeloh, J. A., D. L. Fritz, D. M. Waag, J. M. Hartings, and G. P. Andrews.** 2003. Biodefense-driven murine model of pneumonic melioidosis. Infect. Immun. **71:**584–587.
35. **Jenkin, G. A., T. P. Stinear, P. D. Johnson, and J. K. Davies.** 2003. Subtractive hybridization reveals a type I polyketide synthase locus specific to *Mycobacterium ulcerans*. J. Bacteriol. **185:**6870–6882.
36. **Jha, G., R. Rajeshwari, and R. V. Sonti.** 2007. Functional interplay between two *Xanthomonas oryzae* pv. oryzae secretion systems in modulating virulence on rice. Mol. Plant-Microbe Interact. **20:**31–40.
37. **Jones, A. L., T. J. Beveridge, and D. E. Woods.** 1996. Intracellular survival of *Burkholderia pseudomallei*. Infect. Immun. **64:**782–790.
38. **Karlin, S.** 2001. Detecting anomalous gene clusters and pathogenicity islands in diverse bacterial genomes. Trends Microbiol. **9:**335–343.
39. **Kavanagh, K., and E. P. Reeves.** 2004. Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens. FEMS Microbiol. Rev. **28:**101–112.
40. **Leakey, A. K., G. C. Ulett, and R. G. Hirst.** 1998. BALB/c and C57Bl/6 mice infected with virulent *Burkholderia pseudomallei* provide contrasting animal models for the acute and chronic forms of human melioidosis. Microb. Pathog. **24:**269–275.
41. **Lee, M. A., and Y. Liu.** 2000. Sequencing and characterization of a novel serine metalloprotease from *Burkholderia pseudomallei*. FEMS Microbiol. Lett. **192:**67–72.
42. **Liu, B., G. C. Koo, E. H. Yap, K. L. Chua, and Y. H. Gan.** 2002. Model of differential susceptibility to mucosal *Burkholderia pseudomallei* infection. Infect. Immun. **70:**504–511.
43. **Liu, H., S. Zhang, M. A. Schell, and T. P. Denny.** 2005. Pyramiding unmarked deletions in *Ralstonia solanacearum* shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence. Mol. Plant-Microbe Interact. **18:**1296–1305.
44. **Liu, L., T. Spilker, T. Coenye, and J. J. LiPuma.** 2003. Identification by subtractive hybridization of a novel insertion element specific for two widespread *Burkholderia cepacia* genomovar III strains. J. Clin. Microbiol. **41:**2471–2476.
45. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
46. **Miyata, S., M. Casey, D. W. Frank, F. M. Ausubel, and E. Drenkard.** 2003. Use of the *Galleria mellonella* caterpillar as a model host to study the role of the type III secretion system in *Pseudomonas aeruginosa* pathogenesis. Infect. Immun. **71:**2404–2413.
47. **Moore, R. A., S. Reckseidler-Zenteno, H. Kim, W. Nierman, Y. Yu, A. Tuanyok, J. Warawa, D. DeShazer, and D. E. Woods.** 2004. Contribution of gene loss to the pathogenic evolution of *Burkholderia pseudomallei* and *Burkholderia mallei*. Infect. Immun. **72:**4172–4187.
48. **Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne.** 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10:**1–6.
49. **Nierman, W. C., D. DeShazer, H. S. Kim, H. Tettelin, K. E. Nelson, T. Feldblyum, R. L. Ulrich, C. M. Ronning, L. M. Brinkac, S. C. Daugherty, T. D. Davidsen, R. T. Deboy, G. Dimitrov, R. J. Dodson, A. S. Durkin, M. L. Gwinn, D. H. Haft, H. Khouri, J. F. Kolonay, R. Madupu, Y. Mohammoud, W. C. Nelson, D. Radune, C. M. Romero, S. Sarria, J. Selengut, C. Shamblin, S. A. Sullivan, O. White, Y. Yu, N. Zafar, L. Zhou, and C. M. Fraser.** 2004. Structural flexibility in the *Burkholderia mallei* genome. Proc. Natl. Acad. Sci. USA **101:**14246–14251.
50. **O'Quinn, A. L., E. M. Wiegand, and J. A. Jeddeloh.** 2001. *Burkholderia pseudomallei* kills the nematode *Caenorhabditis elegans* using an endotoxin-mediated paralysis. Cell. Microbiol. **3:**381–393.
51. **Pilatz, S., K. Breitbach, N. Hein, B. Fehlhaber, J. Schulze, B. Brenneke, L. Eberl, and I. Steinmetz.** 2006. Identification of *Burkholderia pseudomallei* genes required for the intracellular life cycle and in vivo virulence. Infect. Immun. **74:**3576–3586.
52. **Rainbow, L., M. C. Wilkinson, P. J. Sargent, C. A. Hart, and C. Winstanley.** 2004. Identification and expression of a *Burkholderia pseudomallei* collagenase in *Escherichia coli*. Curr. Microbiol. **48:**300–304.
53. **Reckseidler, S. L., D. DeShazer, P. A. Sokol, and D. E. Woods.** 2001. Detection of bacterial virulence genes by subtractive hybridization: identification of capsular polysaccharide of *Burkholderia pseudomallei* as a major virulence determinant. Infect. Immun. **69:**34–44.
54. **Redfearn, M. S., N. J. Palleroni, and R. Y. Stanier.** 1966. A comparative study of *Pseudomonas pseudomallei* and *Bacillus mallei*. J. Gen. Microbiol. **43:**293–313.
55. **Rotz, L. D., A. S. Khan, S. R. Lillibridge, S. M. Ostroff, and J. M. Hughes.** 2002. Public health assessment of potential biological terrorism agents. Emerg. Infect. Dis. **8:**225–230.
56. **Schell, M. A., R. L. Ulrich, W. J. Ribot, E. E. Brueggemann, H. B. Hines, D. Chen, L. Lipscomb, H. S. Kim, J. Mrazek, W. C. Nierman, and D. Deshazer.** 2007. Type VI secretion is a major virulence determinant in *Burkholderia mallei*. Mol. Microbiol. **64:**1466–1485.
57. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
58. **Smith, M. D., V. Wuthiekanun, A. L. Walsh, and N. J. White.** 1995. Quantitative recovery of *Burkholderia pseudomallei* from soil in Thailand. Trans. R. Soc. Trop. Med. Hyg. **89:**488–490.
59. **Smith, M. P., T. R. Laws, T. P. Atkins, P. C. Oyston, D. I. de Pomerai, and R. W. Titball.** 2002. A liquid-based method for the assessment of bacterial pathogenicity using the nematode *Caenorhabditis elegans*. FEMS Microbiol. Lett. **210:**181–185.
60. **Stevens, M. P., M. W. Wood, L. A. Taylor, P. Monaghan, P. Hawes, P. W. Jones, T. S. Wallis, and E. E. Galyov.** 2002. An Inv/Mxi-Spa-like type III protein secretion system in *Burkholderia pseudomallei* modulates intracellular behaviour of the pathogen. Mol. Microbiol. **46:**649–659.
61. **Tan, M. W.** 2002. Cross-species infections and their analysis. Annu. Rev. Microbiol. **56:**539–565.
62. **Tuanyok, A., M. Tom, J. Dunbar, and D. E. Woods.** 2006. Genome-wide expression analysis of *Burkholderia pseudomallei* infection in a hamster model of acute melioidosis. Infect. Immun. **74:**5465–5476.
63. **Ulrich, R. L., K. Amemiya, D. M. Waag, C. J. Roy, and D. DeShazer.** 2005. Aerogenic vaccination with a *Burkholderia mallei* auxotroph protects against aerosol-initiated glanders in mice. Vaccine **23:**1986–1992.
64. **Ulrich, R. L., and D. DeShazer.** 2004. Type III secretion: a virulence factor delivery system essential for the pathogenicity of *Burkholderia mallei*. Infect. Immun. **72:**1150–1154.
65. **Vandamme, P., B. Holmes, T. Coenye, J. Goris, E. Mahenthiralingam, J. J. LiPuma, and J. R. Govan.** 2003. *Burkholderia cenocepacia* sp. nov.—a new twist to an old story. Res. Microbiol. **154:**91–96.
66. **Warawa, J., and D. E. Woods.** 2005. Type III secretion system cluster 3 is required for maximal virulence of *Burkholderia pseudomallei* in a hamster infection model. FEMS Microbiol. Lett. **242:**101–108.
67. **Wheelis, M.** 1998. First shots fired in biological warfare. Nature **395:**213.
68. **Woods, D. E.** 2002. The use of animal infection models to study the pathogenesis of melioidosis and glanders. Trends Microbiol. **10:**483–484. (Discussion, **10:**484–485.)
69. **Wretlind, B., A. Bjorklind, and O. R. Pavlovskis.** 1987. Role of exotoxin A and elastase in the pathogenicity of *Pseudomonas aeruginosa* strain PAO experimental mouse burn infection. Microb. Pathog. **2:**397–404.
70. **Zanetti, F., G. De Luca, and S. Stampi.** 2000. Recovery of *Burkholderia pseudomalle*i and *B. cepacia* from drinking water. Int. J. Food Microbiol. **59:**67–72.